

The University of Chicago

**STUDIES ON THE EFFECT OF HIGH
DOSES OF IRRADIATED AND NON-
IRRADIATED ERGOSTEROL
ON THE ALBINO RAT**

A PART OF A DISSERTATION SUBMITTED TO
THE FACULTY OF THE DIVISION OF THE
BIOLOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY
AND PHARMACOLOGY

Year of Convocation
August, 1932

By
JOHN THEOBALD HAUCH

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STUDIES ON THE EFFECT OF HIGH DOSES OF IRRADIATED AND NON-IRRADIATED ERGOS- TEROL ON THE ALBINO RAT ¹

J. T. HAUCH

*Department of Physiological Chemistry and Pharmacology, The University
of Chicago*

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The very earliest studies with irradiated products indicated that they had a toxic action, and likewise the exposure of the animal to light also indicated toxic action. However, later studies led to irregular reports by various investigators. These irregularities were in part due to the fact that some of the investigators reported their dosages in terms of milligrams of ergosterol and not in terms of the therapeutic dose and also because the methods of irradiation used at that time undoubtedly introduced variable potencies and toxicity.

It is not our purpose to discuss all the papers on this subject, but to emphasize only the most important studies on rats. Thus the work of Rosenheim and Webster ('27) showed that 10,000 times the minimum antirachitic dose was not lethal to rats, while the work of Pfannenstiel ('28) and of Kreitmar and Moll ('28), using 'enormous doses,' claimed pathhological findings for several species and also a species variation. Dixon and Hoyle ('28) fed a moderate excess of irradiated ergosterol to rats on a normal bread and milk diet and found no appreciable effect. Similar rats on a synthetic basal diet fed five and eight times larger doses failed to gain weight and on autopsy showed calcium phosphate in the urinary

¹ This work was undertaken on the suggestion of Mead Johnson & Co. who supplied the funds for the first year and who throughout the entire period have supplied us with the ergosterol preparations.

tract. Subsequently ('29) they fed even larger doses and substantiated the above findings. About the same time, Harris and Moore ('28) reported that on a synthetic diet, a dose 1000 times the minimum antirachitic dose was harmless to rats, while 100,000 times overdosage was rapidly fatal. The material used was ergosterol irradiated in an oily medium. They interpret their results to indicate that the irradiated ergosterol itself and not unchanged ergosterol or decomposition products was the factor responsible for the toxic action. These workers claimed to have obtained all the pathological findings reported by the earlier workers. Hoyle ('30), with rats on both synthetic and ordinary bread and milk diets, showed that when a synthetic diet was used he could readily obtain all the striking results of the earlier workers and also confirmed the findings of Harris and Moore. He further showed that if he fed high enough concentrations of irradiated ergosterol on a bread and milk diet he then could also obtain some toxic results although they were not as striking as on a synthetic diet. However, he showed that neither bread alone nor milk alone could prevent this condition, but that the two together acted as a preventative, and therefore concluded that there are two factors which one has to consider in the production of this condition, namely: a) a synthetic diet and, b) an excess of irradiated ergosterol. And finally, he showed that the toxic action was due to a) an excess of irradiated ergosterol and, b) an unknown toxic substance which was much more readily produced when the ergosterol was irradiated in an alcohol solution, being only difficultly produced when the irradiation was carried on in oil. Holtz and Schreiber ('30), working on mice, rabbits and dogs, confirmed the contention of Hoyle and showed that the antirachitic factor and the toxic factor are two separate principles. Furthermore, they showed that the antirachitic activity does not parallel the toxic action in all preparations. Seel ('29) showed that in rabbits large doses of irradiated ergosterol caused a marked loss of weight, emaciation and other cachectic symptoms, and that animals which were rachitic were more

sensitive to larger doses than were normally fed animals. Vara-Lopez ('30) showed that large doses of Vigantol caused changes in the spleen, adrenals and entire reticulo-endothelial system which were peculiar to the Vigantol solution not being given by Vigantol tablets, cod liver oil, or by cholesterol in oil. Goebel ('32) demonstrated that preparations of irradiated ergosterol differing as to industrial source or duration of activation differ also in their toxic effect when fed in massive doses. Bills and Wirick ('30) report experiments running over a longer period of time than the previous work, and showed that 1000 times the therapeutic dose caused just perceptible effects, 4000 times the therapeutic dose was definitely injurious, and 40,000 times the therapeutic dose was strongly toxic. In their work they used both the McCollum 3143 rachitic diet and the stock diet which is used in that laboratory and which cannot be considered as a bread and milk diet, according to Hoyle and his co-workers.

Our work was started in December, 1928. It was our object to maintain throughout our experimental procedures as normal conditions as possible and to limit our observations to only one commercial product which was prepared and furnished by Mead Johnson & Co.

EXPERIMENTAL METHODS

A. Method of assay employed

1. *Line test.* The method of assay used was to place a group of five rats weighing about 45 to 50 gm. on McCollum's diet 3143 for 14 days, at the end of which period the material to be assayed was fed separately to each rat. Two methods were employed in this individual feeding. In the earlier work, the material to be tested was first mixed with a small quantity of the rachitic diet and was then fed to the rats in separate cages and each animal was left in the cage until it had completely eaten its ration. The cod liver oil and earlier stocks of special Acterol were assayed by this feeding method. In the later work, a known concentration of the material in

peanut oil was fed to the animals by means of a stomach tube. This latter procedure seemed to us to be a more accurate measure of the amount actually utilized by the animals. The rats were fed for 10 days on this experimental diet and the bones examined according to the usual line test technic ('22). A therapeutic dose was considered to produce a ++ heal in 10 days.

All the feeding tests involving assay were carried out in a darkened room from which all possible sources of ultra-violet had been eliminated. The animals, when not eating their special diet, were kept in large cages equipped with false bottoms. All cages were thoroughly washed with boiling water and then sterilized in an autoclave once a week. All feed and drinking cups were well washed with hot water daily.

2. *Blood calcium and phosphorus methods.* Blood calcium and phosphorus were determined on two rats of each group on the fourteenth day of the experiment and again just before the rats were killed on completion of the assay. The blood was drawn by heart puncture, which yielded from $1\frac{1}{2}$ to 2 cc. of blood without seriously affecting the rats. Because of the small volume of blood available, it was necessary to modify the regular methods and to use whole blood instead of serum. For inorganic phosphorus, the Fisk-Subbarow method ('25) was modified so that the blood and reagents were used in one-fifth quantities. For calcium, a modification of the Roe-Kahn procedure ('26) for calcium was devised so that the equivalent of 0.5 cc. of whole blood was used for duplicate estimations. In both determinations, a microcolorimeter was used.

B. Routine procedures in the high dosage experiments

1. The animals were fed either the regular stock diet or the rachitic diet plus a definite quantity of Superactrol per rat per day. This extra ration was fed in three different ways. In the earlier work, it was mixed with the regular stock diet, while in the later work, where it was desirable to use higher

doses, we first used a carefully calibrated medicine dropper which was eventually replaced by a graduated stomach tube.

2. All animals were weighed weekly and the growth curves plotted.

3. The behavior of the animals was observed and a careful record of any irregularities kept.

4. Records were kept of matings and of the weight, size and condition of all litters.

5. Animals sacrificed or found dead were carefully autopsied. The organs of the chest and abdomen were carefully examined grossly. The urinary tract was usually dissected out intact and examined under the dissecting microscope for possible calculi. The following organs were carefully weighed: thymus, heart, lungs, liver, spleen, kidneys, and, in the males, the testes.

6. For microscopic study sections were prepared from the thyroid, thymus, aorta, heart, lungs, liver, stomach, duodenum, spleen, kidneys and gonads.

EXPERIMENTS AND RESULTS

A. Standardization of cod liver oil and Acterol

Our assays showed that 0.3 cc. per rat per day of a solution composed of one part of cod liver oil and twenty-nine parts of peanut oil gave a good (++) cure in 10 days. Control experiments showed that the peanut oil used had no detectable activity. On the basis of the statement that the Acterol used was one hundred times as potent as the cod liver oil, we fed a solution of Acterol in which the Acterol was one one-hundredth the concentration of the cod liver oil fed above. This gave us a cure which was slightly better than ++, but which we believed to be within the limits of biological assay. In another assay on a still more potent solution, we used the stomach tube method and found that the strength of the solution was exactly what it was calculated to be. This latter material was a solution of Mead-Johnson's Viosterol which had a potency of 10,000 D $\pm$ 17 per cent. The studies on the cal-

cium and phosphorus showed that there is a more or less distinct relation between the blood and bone pictures. In severe rickets, on the fourteenth day of the rachitic diet, the calcium varied from 6.5 to 7.5 mg. per 100 cc. of whole blood, while the inorganic phosphorus values ran from 1.5 to 3.0 mg. per 100 cc. After being on a curative ration for 10 days following the 14 days on a rachitic diet, the values were found to range from 8.0 to 9.5 mg. per 100 cc. for calcium, and from 4.0 to 6.0 mg. per 100 cc. for phosphorus. When the test ration was not potent enough to give any cure, the values for calcium and phosphorus became even lower than they were on the fourteenth day of the rachitic diet.

B. High dosage experiments

Series 1. Feeding experiments with high dosages of irradiated ergosterol given to albino rats on a standard normal diet.

(a) Eighteen normal albino rats, 5 weeks old, placed on the normal stock diet, were divided into four groups containing about the same number of males and females. Each one of these groups was fed, in addition to its normal diet, either a special dose of Acterol (1:100) or an equivalent amount of non-irradiated ergosterol in peanut oil. The Acterol or ergosterol was fed to each animal individually once each day. These additional diets were made up in the following way:

Group I received a diet calculated to contain one hundred times the therapeutic rat dose of Acterol. In all our calculations we adopted the therapeutic dosage as suggested by Mead Johnson & Co. According to their standards, about 1/80 gm. of cod liver oil per rat per day gives a good ++ line test in 5 days, while Acterol is one hundred times as potent. On this basis the diet of group I was made up by taking 100 cc. of Acterol and diluting it with 91 cc. of peanut oil. We estimated that one standardized drop of this mixture would be equivalent to one hundred times the therapeutic rat dose of Acterol.

Group III received an extra ration of four drops of undiluted Acterol (1:100) per rat per day, which is equivalent to approximately eight hundred times the therapeutic rat dose of Acterol.

Groups II and IV received diets containing unirradiated ergosterol in amounts equivalent to the irradiated ergosterol present in the Acterol of the diets of groups I and III, respectively. Based upon the statement of Mead Johnson & Co., that Acterol contains 50 mg. of ergosterol in 140 cc. of oil, the diet of group IV was 4 drops per day of a solution of 50 mg. unirradiated ergosterol completely dissolved in 140 cc. of peanut oil. Group II received as an extra ration 1 drop of a mixture of 100 cc. of the above solution of unirradiated ergosterol and 91 cc. of peanut oil.

This experiment was continued over a period of 42 weeks (3/4/29 to 1/3/30). The weights of the animals in these four groups showed that growth was fairly normal. No distinct gross pathology was observed. Several of the rats in group I showed pathological changes in the lungs, but this was diagnosed as pneumonia, a very common rat ailment, and could, therefore, not be attributed to the Acterol fed. All the females in group I and one female in group III failed to reproduce which at first led us to believe that a factor of sterility might be involved. This experiment has been repeated many times with animals receiving ten and a hundred times as much of the same lot of Acterol without producing any similar conditions, and we therefore conclude that the failure in reproduction was due to some factor other than Acterol.

(b) The second experiment under this general heading was started with ten 6-month old rats. These animals were fed a normal diet with the exception that the animals in group V received an extra individual ration consisting of ten thousand times the therapeutic rat dose of Acterol, while the four rats of group VI were fed an extra daily ration of unirradiated ergosterol in peanut oil equivalent to the concentration of ergosterol in the diet of the rats in group V. The actual

amount of extra material fed in this case was 7 drops of Superacterol which is six times as potent as Acterol, according to Mead Johnson & Co. assay. This experiment was carried on for a period of 3 years and 15 weeks (4/5/29 to 7/20/32). As the first and second generation animals were obtained they were immediately, on weaning, placed on the same test diet as their parents. In some of the later work the young were fed the test diet directly by means of a medicine dropper beginning at the first or second week after birth.

In summing up the work on these groups, we can say that there was very little if any difference in the growth of the two groups or their offspring. Several of the young in group V died shortly after weaning, but this may be explained by the fact that in these cases the mother had been mated too young and the whole litter was not up to the standard. Furthermore, the later work, when greater care was taken in mating, shows no such picture. Throughout this work no gross or microscopic pathology was observed except that which was due to pneumonia. Even those young which received this large dose of Superacterol when only a week old appeared perfectly normal in all respects upon weaning. Four of these young animals, of the fourth generation, were selected and placed on an extra ration of 15 drops of a new lot of Superacterol which had a potency of 1000 D. These animals received approximately 36,000 times the therapeutic rat dose over a period of 2 years and 21 weeks (2/21/30 to 7/20/32), and seemed to be normal in all respects. The three females all gave birth to normal litters. One of the females was killed during the thirty-first week of the experiment and a careful study of her tissues showed no pathology. During the forty-second week of this experiment the three remaining animals were placed on 20 drops of Superacterol which was equivalent to approximately 50,000 times the therapeutic rat dose. The male in this group was killed in the fiftieth week of the experiment and again the tissues were studied for evidences of pathological lesions with no results. The second female of this series died during the sixty-sixth week of the

experiment and again there were no signs of gross or microscopic pathology. The last animal, female 153, died in the sixty-ninth week of the experiment, while blood was being drawn from the heart by means of heart puncture.

Eighteen of the fifth-generation animals from the litters of the three fourth-generation females were observed over a period of 43 weeks (10/9/30 to 8/25/31), while receiving approximately 36,000 times the therapeutic rat dose and at no time did they exhibit any pathological symptoms. Twelve young animals of the sixth generation were observed under the same conditions over a period of 18 weeks (4/25/31 to 8/20/31) without showing any toxic symptoms. This is the shortest period of observation for any of the animals in this series. The total number of animals in this experiment was 127 and they were divided in the following manner: 10 first-generation adults, 48 second, 35 third, 4 fourth, 18 fifth, and 12 sixth-generation young animals.

(c) The third experiment under this general heading was started with five normal animals 4 weeks of age. These animals were fed a normal stock diet which was made up so that it contained 25 mg. of irradiated ergosterol per 10 gm. of original diet. This ergosterol was irradiated in the dry state by a Cooper-Hewitt mercury vapor lamp; the time of irradiation was 35 minutes at a distance of 40 cm. An accurate record of the food consumption of each rat was kept and it was found that on an average each rat ate 10+ gm. of food per day which meant that each animal was getting 25+ mg. of irradiated ergosterol per day. An assay of this irradiated ergosterol showed that it gave a good ++ cure in 10 days when fed in doses of 0.001 mg. per rat per day. Thus the animals in this experiment were receiving 25,000 times the therapeutic rat dose. These animals were kept under observation for 23 weeks (8/12/29 to 1/24/30), and showed absolutely no evidences of hypervitaminosis. The offspring of these animals were also put on the same diet and observed for 10 weeks (1/13/30 to 3/28/30), and likewise gave no signs of abnormality.

Series 2. Feeding experiments with high doses of irradiated ergosterol given to albino rats on a rachitic diet. (a) The following experiment was designed to study the possible toxic effects of excessive doses of Acterol on animals which were on an otherwise rachitic diet. Sixteen rats, 4 weeks of age, were placed on the McCollum diet 3143 for a period of 14 days. At the end of that time ten of them were fed in addition a quantity of Acterol equivalent to ten thousand times the therapeutic dose. The remaining six rats were fed on a control diet of unirradiated ergosterol in peanut oil. With the exception of female 22 all the animals on Acterol showed a fairly normal growth for the first 5 weeks, although the rate of gain was slightly lower than normal. Female 22, the exception to the above, commenced to lose weight as soon as she was put on the curative diet and finally in the fifth week on curative diet she was killed and an autopsy performed, the report of which is herewith given:

Although this animals had lost 10 gm. in weight (80 to 70 gm.) during the 31 days on the test diet, at autopsy no definite pathology was identified. No calcium deposits were noted in any of the tissues nor were there any proliferative changes or necrosis such as have been described by other workers as preliminary to the pathological calcification which they have reported. No calculi were present in the urinary tract. The great vessels were elastic and were not sclerosed or calcified.

After the fifth week the remaining animals in this group began to show distinct fluctuations in growth. This was true in all cases except that of female 34 which showed a constant though slow gain. Male 28, although he had been gaining weight slowly, suddenly developed a distaste for his food and refused to eat. He next developed a marked diarrhea and an enormous distention of the bladder and finally, having eaten very little for 2 or 3 days, he went into distinct tetanic convulsions in which he died. The autopsy showed these conditions:

This animal had a bladder which measured 20 x 20 x 25 mm. Both ureters were distended and the kidneys were enlarged.

No calculi were found. The kidneys weighed together 2.33 gm., about double the normal weight for this size animal. The spleen seemed abnormally small. The microscopic picture was that of a hydronephrosis. No line of calcification at the junction of cortex and medulla was seen as reported by Dixon and Hoyle ('28). All other tissues were normal.

In order to make a comparative pathological study, female 132 of this group was sacrificed as was also a control female of the same age. Studies by various different staining techniques for showing calcium, which will be described in a subsequent paper on the microscopic findings, showed no differences between the two animals which could be classified as pathological.

Male 23 showed a distinctly poor growth curve although his appetite was good. His total gain in weight at the peak of his growth curve in the twenty-third week, was only 49 gm. Besides this he showed a distinct prolapse of the penis. The penis, hanging out from the body, had the appearance of a loose bluish-colored appendage of the skin. The other male in this group, 35, though underweight, seemed to be normal in all respects.

The three animals in this control group on unirradiated ergosterol showed no great differences from the above group. Female 36 which showed an irregular growth curve died during the twenty-second week and the following gross changes were observed:

There was some thinning of the hair especially over the back, moderate emaciation and considerable paleness of the tissues except the lung, which was bright red. The liver showed the lobular markings quite prominently and an area at one margin $5 \times 3 \times 2$ mm. in which the liver tissue appeared pale yellowish and caseous. This was surrounded by a dark hemorrhagic zone about 1 to 2 mm. wide. All other tissues examined appeared normal although no thymus tissue could be identified.

Of the two males in this control group, male 38 exhibited the symptoms listed above of flaccid penis, etc., and male 42

seemed quite normal in all respects except in weight. However, this male showed a better and more consistent gain in weight than any animal in either group.

In reviewing the work on this group which was in progress for 51 weeks (2/27/29 to 2/21/30), we find that all the animals in the control group died except male 42, which seemed to be perfectly normal in all respects. In the group receiving the Acterol all animals except female 34 and male 35 died. These two animals appeared almost normal although they were not quite as sleek looking as our stock animals. Female 34 had one litter of five out of which three died before they were weaned. It is our impression that the irregularities in growth and development in the animals in this group were due to the deficiency of the diet rather than to any effects of the ergosterol feeding.

The most striking observation, however, was the fact that none of the animals, in the control group, which came to autopsy, showed active rickets although they had been on a vitamin D free diet. There are several reasons which might have been the cause of this situation. In the first place, three of the animals in this group died and were eaten by their litter mates before we were aware of their death. In this way the phosphorus and the small amount of residual vitamin D in the tissue of the dead animals was made available to the survivors. Next, those which were autopsied showed a slight loss of weight before death or else had not shown any gain for several weeks. It is known that in order to show a typical rachitic bone picture at autopsy an animal must have shown consistent growth throughout the experimental period. Another factor which may have been of some importance was the fact that after the twenty-third week of the experiment, the animals were not kept in a completely darkened room, although they were never exposed to the direct sunlight. Then, finally, in feeding such a high concentration of ergosterol it might be possible that there was a sufficient contamination by active material to cause the animals to show a normal bone picture.

Series 3. Feeding experiments with extremely high doses of irradiated ergosterol given to albino rats on a normal diet, with and without potassium iodide, and on a rachitic diet without iodide. (a) The negative results which were obtained in the above experiments led us to start an experiment in which extremely large doses were given to the animals in an effort to see whether we could obtain any of the toxic symptoms reported by others. In this series, we used the Mead-Johnson Viosterol which had a potency of 10,000 D. All test materials were fed by stomach tube. We started with fifty-two albino rats which were divided in the following manner:

Group VII. Twenty-seven animals received the normal stock diet plus 93,000 times the dosage of Viosterol.

Group VIII. Twenty-five animals received as an added daily ration 93,000 times the therapeutic rat dose of Viosterol and also various amounts of potassium iodide. The iodide was added in this case because of the claims made by Simonnet and Tanret ('30), who maintained that the addition of large quantities of iodide prevent the calcification due to excessive doses of irradiated ergosterol.

Group IX. Twenty controls, ten of which received a quantity of peanut oil equivalent to the amount received by the experimental animals, and the other ten received an amount of potassium iodide equivalent to the amount fed to the test animals.

This experiment was continued over a period of 37 weeks (11/6/31 to 7/21/32). As far as the gross condition of the rats is concerned, the feeding of the iodide had neither a deleterious nor advantageous effect. The animals seemed normal in all respects and no gross pathology was observed.

It was, however, felt desirable to carry this experiment on a little further, so three of the healthiest males of group VII were taken and fed an amount of Viosterol equivalent to the 465,000 times dosage. Some very striking results were now obtained. Two of these animals died on the fifth day of feeding and the third showed a distinct loss in weight, a

general emaciation, a slight diarrhea and a definite loss of appetite with an associated general apathy. Two of the animals showed a distinctly bloody urine and the last animal to die showed very marked evidences of calcification in the aorta. This striking finding led to a continuation of the experiment and this was done by starting nine young animals on this high dosage. In this case three animals died during the first week, four during the second week, and the rest during the third week.

(b) In order to satisfy ourselves as to the validity of these results we next treated twenty-seven normal adults as follows:

Group X. Thirteen normal females, 9 months old, who had all had one normal litter, were so divided that nine of them received the 465,000 times dosage of Viosterol, while the remaining four received the 93,000 times dosage.

Group XI. Seven normal females, and seven normal males, all 3 months of age, were so divided that five females and six males received the 465,000 times dosage, while the balance of two females and one male received the 93,000 times dosage of Viosterol.

All animals on the 465,000 times dosage died within 5 weeks; 11 females and 2 males in the first, 2 females and 3 males in the second, 1 male in the fourth and 1 female in the fifth week. All developed a slight diarrhea, loss of weight, a flabby emaciated musculature, inactivity and loss of appetite. Most of them showed a bloody nasal discharge which appeared to be associated with a difficulty in breathing. All of the rats on the 93,000 times dosage survived during this period although some of them did not appear to be normal and had lost considerable weight. The blood from several of these animals was analyzed for calcium and inorganic phosphate, and no significant rise was noted, which is in agreement with the report of Jones, Rapoport and Hodes ('30).

(c) A third experiment under this general heading was now started and this involved the use of eighty-four normal animals of approximately 4 weeks of age. These animals were divided into three major groups each of which was again

subdivided into three smaller groups according to the following scheme:

Group XII. Thirty animals were placed on a rachitic diet for a period of 2 weeks, at the end of which time they were kept on the same basic diet and received in addition 46,500, 93,000 and 465,000 times the therapeutic rat dose of Viosterol, respectively.

Group XIII. Twenty-seven rats were placed on a rachitic diet for a period of 14 days at the end of which time they were placed on a normal diet and divided into the three sub-groups listed above.

Group XIV. Twenty-seven animals were placed on a normal stock diet for a period of 2 weeks, at the end of which time they were divided into the three sub-groups referred to above, but continued on the normal basal stock diet.

In this experiment, we wished to determine three things, 1) were the animals more resistant to this toxicity when on a McCollum vitamin D free diet than when on a normal diet; 2) what was their reaction when rachitic and then replaced on a normal diet plus the overdosage of irradiated ergosterol; and, 3) the difference between the various levels fed under the different conditions of the experiment. The results obtained under these conditions were quite clear cut and definite. All of the animals receiving the 465,000 times dosage with McCollum's vitamin D free diet were dead by the sixth day following the first dose of irradiated ergosterol. These animals all showed evidences of emaciation with an extensive loss of weight, all exhibited signs of diarrhea and all had a bloody discharge from the nose. At autopsy no gross pathology of the organs was observed. The animals receiving the 93,000 times dosage did not show the extreme symptoms listed above, but they did show a steady loss of weight and by the end of the third week were so weak that they could hardly move about. All of these animals died or were killed during the fourth week. Some of these animals had a slight diarrhea and all had a more or less bloody nose. The main gross autopsy findings were that in all but two of the animals the

liver and kidneys were a pale grayish brown color speckled with darker brown dots. In female 500 the lung was full of caseous nodules while in male 497 the spleen was very friable. The animals receiving the 46,500 times dosage appeared normal in growth and appearance for the first 2 weeks, and then all but four of them also began to lose weight, although their loss was only gradual. These animals were all killed during the fifth week of the experiment and we were unable to observe any gross pathology other than the loss of weight.

In the group of animals which was transferred from the rachitic to the normal diet at the end of the second week, several characteristic findings were observed. Of the animals receiving the 465,000 times dosage, two died during the first week, seven during the second week and the remaining animal died during the third week of the experiment. Here again the animals had lost much weight and at autopsy we observed that the kidneys were of a very pale grayish brown color, speckled with darker brown dots, and on cutting through the kidneys it was seen that this was the color of the entire cortex. The thymus in all these animals was quite small and the adrenals appeared larger than those of normal animals. The hearts of these animals showed a yellowish mottling against the background of the darker myocardium. The animals which were receiving the 93,000 times dosage appeared quite normal during the first week of the experiment and with the exception of two animals which lost slightly in weight, appeared quite normal during the second week. All of these rats were killed during the third week of the experiment and no gross evidences of pathology were observed. Those animals receiving the 46,000 times dosage appeared perfectly normal and when killed during the third week showed absolutely no symptoms of any gross pathology.

The groups receiving a normal diet from the time of weaning, and which were 2 weeks later placed on the 46,500 and 93,000 times dosage were normal in all respects and at autopsy during the third week gave no evidences of any gross pathol-

ogy. The animals receiving the 465,000 times dosage again showed all the symptoms listed in the previous two groups on this level of Viosterol, namely, pale speckled kidneys, mottled heart, small thymus and large adrenals. All but one of these animals were dead by the end of the second week of the experiment and this animal was killed during the third week. The arch of the aorta of this animal (male 564) showed signs of calcification. In order to assure ourselves that we were observing all the gross pathology, we killed two groups of three animals each, consisting of litter mates on the three levels of irradiated ergosterol fed. The tissues of these animals were then compared grossly with each other and with the tissues of a normal untreated animal of the same age. When this was done it was seen that the thymus of the animals on the 465,000 times dosage was less than one-fourth the weight of the thymus in the other treated animals and in the normal control. In fact, in the subsequent animals brought to autopsy this relationship was in most cases much more pronounced. This comparative study also emphasized the previously noted difference in the size of the adrenals and also definitely brought out the fact that the myocardium of the highest dosage animals was affected in some manner by the production of slightly elevated areas of a yellowish mottling which did not appear to correspond to the major vessels of the myocardium. Furthermore, this study substantiated our belief that, at least from a consideration of gross pathology, the animals receiving the 46,500 and the 93,000 times dosage were in all respects normal. Finally, in order to determine whether the change in the thymus was an atrophy or merely a cessation of growth, several normal animals, of the age of the experimental ones at the time when they were first fed ergosterol, were killed and it was observed that in all these cases the thymus of the untreated animals was two to three times larger than the thymus of the highest dosage animals and that in all instances the thymus was smaller than the thymus of the animals receiving the 46,500 and 93,000 times dosage, respectively, or than the thymus of the older normal controls.

Series 4. Experiments on the protection of the young against rickets. With so many young animals available from the other experiments in progress, we thought it would be interesting to check the findings of Bills and Wirick ('30) as to the influence of activated ergosterol in the mother's diet on the resistance of the young to rickets. Seventy-six animals were used in this work. These animals were from mothers receiving 10,000 times the therapeutic rat dose of irradiated ergosterol and were placed on McCollum's rachitic diet 3143 at weaning. At the end of a 3-week period the animals were killed and studied by the line test and blood analyses for rickets. In all cases, there was only a slight degree of rickets.

Another experiment involving thirty-seven rats was run. In this experiment the mothers were receiving an extra ration of 10,000 times the therapeutic rat dose of irradiated ergosterol and immediately after birth the young were given to a foster mother on normal diet. All of these animals showed the characteristic rachitic blood analyses and line test after having been on the rachitic diet for 3 weeks. These results definitely confirm the findings of Bills and his co-workers ('30).

DISCUSSION

From the data presented it seems clear that rats when fed a normal bread-milk diet are not affected by irradiated ergosterol if given 46,500 times the therapeutic rat dose. When similar animals are given 93,000 times the therapeutic rat dose of irradiated ergosterol some toxic symptoms are observed. The effect of this dosage is, however, quite different on young and adult animals. When young animals are given this amount of irradiated ergosterol, they show no immediate symptoms; in fact, they develop quite normally and it is only as they approach the age of 9 to 12 months that they begin to manifest any symptoms of toxicity and these are not extremely serious, having in no case in our series led to the death of the animal. When 93,000 times the therapeutic rat dose of irradiated ergosterol is fed to animals 3 and 9 months old, respectively, there is an immediate loss

in weight which is much more pronounced in the older animals. In some cases this dosage led to death of the animals with the same gross pathological findings as are observed in animals on a higher dosage. It is quite significant to note that there is a definite difference in the reaction of different adult animals when on this level of irradiated ergosterol. This is very strikingly brought out in the seven rats on this dosage in groups X and XI; of these, two rats, 675 and 619, died while the remaining animals appeared quite normal except for slight loss in weight. This experiment also shows the difference between the reaction due to age, for the animals which were 3 months of age lost very little weight and none of them died while the 9-month-old animals lost weight steadily and two of them died.

When 465,000 times the therapeutic rat dose of irradiated ergosterol was fed to either young or adult animals there was an almost immediate toxic response. This reaction was characterized by the following symptoms: immediate loss of weight, the appearance of diarrhea and of a bloody discharge from the nose, the development of a muscular flabbiness, a definite loss in appetite and a very marked emaciation. When brought to autopsy these animals showed definite gross pathology. There were varying degrees of calcification of the aorta, the kidneys were a very pale grayish color and speckled with darker brown dots, the thymus was atrophied, the adrenals were larger than in the normal, and the myocardium showed a grayish yellow mottling. Here again we can see the greater susceptibility of the adult animals to these high dosages. Seventeen of the adult animals fed on 465,000 times the therapeutic rat dose of irradiated ergosterol and a normal diet were dead by the end of the fifth day, one lived for 2 weeks, while the last two rats lived for 3 weeks. Of the nineteen young animals on this level of irradiated ergosterol and a normal diet only three were dead at the end of the first week while the other sixteen died or were killed between the thirteenth and seventeenth days of the experiment.

Certain workers (Kreitmair and Hintzelmann, '28; Krietmair and Mell, '28, and Pfannenstiel, '27, '28) have also reported the atrophy of the spleen to be one of the results of excessive doses of irradiated ergosterol, but we could not confirm this finding. The weights of the spleens in our animals varied considerably and at times we thought we were confirming this finding. However, when we selected the records of all the animals receiving irradiated ergosterol for which there was a control rat of approximately the same weight, we found that the total weight of the spleens of the animals receiving irradiated ergosterol was 0.37 per cent of the total gross weight, while the total weight of the spleens of the control animals was 0.38 per cent of the total gross weight. From these facts we feel justified in saying that we have not the slightest evidence of any splenic atrophy in our rats. Perhaps the most consistent and outstanding pathology we observed was in the thymus. In the eighty-four animals in groups XII, XIII and XIV, the sixty-five animals on the 46,500 and 93,000 times dosages of irradiated ergosterol as well as four normal controls, had a thymus anywhere from three to four or more times as heavy as any of the animals on the 465,000 times dosage. In fact, in many cases the thymus of the highest dosage animals was so small as to be almost unrecognizable and unweighable.

Many investigators have reported the finding of calculi in the urinary tract which have given rise to hydronephrosis. In our series, we had one rat receiving 10,000 times the therapeutic dose of irradiated ergosterol and McCollum's rachitic diet 3143 which developed hydronephrosis due to an obstruction in the bladder or lower down. We were, however, unable to locate a calculus although it may have been present. Another rat had a mild distention of the bladder and a small calculus 1 mm. in diameter was found at the urinary trigone. However, the largest calculi we found were in a control animal on a rachitic diet. There were two of them, each of which measured $3 \times 3 \times 6$ mm. They were crystalline in structure and gave a strong test for calcium, but only a very faint phosphate test.

Brown and Shohl ('30) claim that animals on a rachitic diet and receiving large overdoses of irradiated ergosterol (Vigantol) are more resistant to the toxic effects than animals on a normal diet. We cannot subscribe to this claim, for in our work we found that the animals on a rachitic diet showed the toxic effects much more rapidly and at a lower level than those on a normal diet. Of the animals in group XII, the ten animals receiving McCollum's rachitic diet 3143 and 465,000 times the therapeutic rat dose of irradiated ergosterol were all dead by the end of the first week, and the ten animals receiving the same diet and 93,000 times the therapeutic rat dose of irradiated ergosterol either had died or were killed by the end of the fourth week because of their extremely weak condition. In contrast to this only three of the animals in groups XIII and XIV receiving the 465,000 times dosage died during the first week, while all but two of the remainder died or were killed during the second week and the last two during the third week. Furthermore, of the eighteen animals in these groups receiving the 93,000 times dosage, only two animals showed a very slight loss of weight at the end of the second week and these two animals were from group XIII and had been on a rachitic diet during the first 2 weeks of the experiment. The other animals were normal in all respects. The difference in these findings may be due to the fact that the rachitic diet used in our work was a much more severe diet than that used by Brown and Shohl. However, in their first paper ('30) they compare animals which had been on a rachitic diet plus irradiated ergosterol for 6 days with animals which had been on a normal diet plus the same amount of irradiated ergosterol for 11 days and we feel that such a comparison is not warranted. In their second paper ('30) these workers do not present enough clear cut experimental evidence to uphold their contention that on a rachitic diet the toxic effects are not as pronounced as on a normal diet.

A detailed discussion of the gross and histological specimens of all the experimental animals will appear in a later publication.

SUMMARY AND CONCLUSIONS

1. Commercial preparations of irradiated ergosterol of exceptional potency were standardized by the McCollum line test and found to possess the claimed activity.

2. Ten albino rats were placed on a stock diet plus 100 to 800 times the therapeutic rat dose of this preparation for 36 weeks without observing harmful effects.

3. The progeny of 48 second, 35 third, 4 fourth, 18 fifth and 12 sixth generation rats were placed on the same stock diet plus doses up to 50,000 times the curative dose without observing harmful effects. The sixth generation animals were treated for 18 weeks, all others were treated for longer periods.

4. Pure ergosterol was irradiated in the solid form by a Cooper-Hewitt mercury arc lamp and fed to albino rats and their offspring in amounts up to 25,000 times the curative dose for 10 to 23 weeks without harmful effects.

5. Albino rats were placed on the McCollum 3143 diet with and without the commercial preparation in amounts up to 10,000 times the therapeutic rat dose. Although most of the animals died in less than 30 weeks, no true hypervitaminosis could be detected.

6. Young and adult albino rats were kept on a stock diet including bread and milk, plus 46,500 times the therapeutic rat dose for 3 weeks without harmful effects.

7. Young albino rats were placed on a stock diet including bread and milk plus 93,000 times the curative dose for 12 months when some of them showed loss of weight. Adult animals under the same conditions showed immediate loss of weight and in some cases died with pathological findings similar to those indicated below.

8. Young and adult albino rats were placed on a stock diet including bread and milk plus 465,000 times the therapeutic rat dose with the appearance of immediate toxic effects. There were immediate loss of appetite and weight, bloody discharge from the nose, diarrhea, marked muscular flabbiness, and gross pathological changes involving enlarged supra-

renals, atrophied thymus and abnormal appearance of the kidney and heart. The adult animals were again more susceptible than the young ones.

9. Rachitic diet was substituted for the stock diet on the 46,500, 93,000 and 465,000 dosage experiments on young animals. This change made the animals more susceptible.

10. Potassium iodide was added on the dosages of 93,000 times the curative amount without changing the picture.

11. Young from mothers on 10,000 times the therapeutic rat dose were weaned and placed on the rachitic diet. Only very slight rickets developed. Similar young, but nursed by stock diet foster mothers, developed typical rickets.

12. These results indicate that the toxic effects observed by others on relatively low dosages of irradiated ergosterol are due to the presence of a toxic substance other than the true antirachitic agent in appreciable amounts in the preparations tested. Whether the toxic effects observed by us on our commercial preparation when given in the very high doses are due to a trace of this hypothetical by-product or to the vitamin D substitute remains to be determined.

13. Our results again indicate that the toxicity of a given irradiated ergosterol preparation is also determined in part by the character of the diet. In general, the more complete and better balanced diets act in a more protective manner.

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